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Reversed-Phase High-Performance Liquid Chromatographic Separation of Wheat Proteinaceous Inhibitors of Insect and Mammalian α-Amylases[1]

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ABSTRACT

A heat-treated 0.15*M* NaCl extract of wheat flour was separated into four proteinaceous fractions by ammonium sulfate precipitation. Each fraction had a distinct composition of polypeptides with apparent molecular mass of approximately 14 kDa and a distinct inhibitory spectrum of activity against human, porcine, and insect (confused flour beetle and rice weevil) α-amylases. More than 10 peaks in each fraction were resolved by reversed-phase high-performace liquid chromatography (RP-HPLC) with mixtures of acetonitrile and H_2O as the mobile phase. RP-HPLC separations revealed more heterogeneity in wheat α-amylase inhibitors than was apparent from either nondenaturing or denaturing sodium dodecyl sulfate gel electrophoresis. RP-HPLC fractions were characterized further by polyacrylamide gel electrophoresis and by assaying inhibitory activity against the four different α-amylases. At least five different HPLC fractions were found to be selective inhibitors of insect α-amylases, some of which belong to the 0.28 family of inhibitors. Combining certain RP-HPLC fractions resulted in dramatic increases in inhibitory activity. These results suggest that RP-HPLC resolves the oligomeric inhibitors into their monomeric forms and that both the activity and selectivity of the inhibitors can be influenced by recombination of individual polypeptides.

Proteinaceous inhibitors of plant, microbial, insect, and/or mammalian α-amylases are found in a number of cereals, legumes, and other plants. Their properties have been reviewed recently by Silano (1987) and Garcia-Olmedo et al (1987). Both exogenous and endogenous α-amylase inhibitors are present in wheat (*Triticum aestivum*). Inhibitors of exogenous α-amylase, which inhibit mammalian, insect, and microbial α-amylases, may play a role in preventing insect and microbial attack (Yetter et al 1979, Powers and Culbertson 1982, Garcia-Olmedo et al 1987); inhibitors of endogenous α-amylase may regulate activities of cereal α-amylases to prevent starch depletion until seed germination (Mundy et al 1984, Weselake et al 1985, Warchalewski 1987, Abdul-Hussein and Paulsen 1989). The possible antinutritional effects of these inhibitors on humans and other animals are of potential importance, particularly in light of some inhibitors' high stability to either heat or acid treatment (Silano 1987). The studies presented in this article focused on the isolation and characterization of exogenous amylase inhibitors from wheat.

Because of the importance of wheat in agriculture and human nutrition, wheat α-amylase inhibitors have been studied extensively. The wheat albumin fraction contains a heterogeneous group of proteins that can inhibit α-amylases from different sources (Silano 1987). Some of the heterogeneity is due to the hexaploidy of the wheat genome (Garcia-Olmedo et al 1987, Gomez et al 1989).

The multiplicity and multimeric nature of wheat α-amylase inhibitors have made it difficult to obtain chemically homogeneous inhibitor polypeptides. Gel filtration and ion exchange chromatography are techniques commonly used in such purifications of wheat α-amylase inhibitors (Baker 1987, Silano 1987). When applying these methods, one can use solvents that should minimize any chance of denaturing (and inactivating) the inhibitors. In recent years, reversed-phase high-performace liquid chromatography (RP-HPLC) has been used in separating and characterizing cereal proteins (Bietz 1986). The value of this technique stems from its inherent resolving power and from the fact that it is complementary to other separation methods. However, organic solvents used in RP-HPLC are potentially denaturing to many proteins.

In this study, we exploited RP-HPLC in fractionating wheat α-amylase inhibitors. It seems that most of these inhibitors survive exposure to the solvent conditions, which include both heptafluorobutyric acid (HFBA) and acetonitrile in the mobile phase. Our results indicate that RP-HPLC is a powerful tool for separating wheat α-amylase inhibitors. Oligomeric inhibitors apparently are dissociated into monomeric polypeptides in the mobile phase, and the chromatography separates individual polypeptide chains. We have determined the inhibitory activities and selectivities of the monomeric wheat proteins and some combinations of them.

MATERIALS AND METHODS

Extraction and Fractionation of Wheat Albumins

Newton wheat kernels were kindly provided by R. C. Hoseney, Department of Grain Science, Kansas State University, Manhattan. Wheat flour (100 g) was extracted with 300 ml of 0.15*M* NaCl for 1 hr at room temperature. After centrifugation (17,400 × *g* for 1 hr), the supernatant (crude extract) was heated in boiling water for 10 min and centrifuged. We call the soluble material after heating the "heat-soluble proteins." This material was further separated by ammonium sulfate precipitation into four fractions. The fractions that precipitated at 0–0.5*M*, 0.5–1.0*M*, 1.0–1.5*M*, and 1.5–2.5*M* ammonium sulfate were designated AS 0–0.5, AS 0.5–1.0, AS 1.0–1.5, and AS 1.5–2.5, respectively. Ammonium sulfate precipitates were suspended in deionized water and dialyzed thoroughly against water at 4°C. After centrifugation, supernatants were freeze-dried, stored at −20°C, and subsequently used for RP-HPLC.

Reversed-Phase High-Performance Liquid Chromatography

RP-HPLC was conducted using a Beckman model 332 liquid chromatographic system (Beckman Instruments, Inc., Fullerton, CA) at room temperature (25°C) with a 4.6- × 250-mm Vydac 5-μm C-18 column (300 AÅ of pore size) (Alltech Associates, Inc., Deerfield, IL). HFBA from Pierce Chemical Co., Rockford, IL, was used as the ion-pairing reagent. Solvent A was 0.1% HFBA in water, and solvent B was 0.1% HFBA in acetonitrile. All solvent components were HPLC grade. Water was prepared with a Milli-Q water system (Millipore Corporation, Bedford, MA).

Ammonium sulfate fractions, prepared as described above, were dissolved in water, mixed with equal volumes of solvent A, and filtered through a Millipore membrane (0.22 μm) (Millipore Corporation). About 0.5 ml of each sample was injected. A gradient program (flow rate = 1 ml min^{-1}) was used to increase solvent

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B in the mobile phase to 35% in 15 min and then to 43% in 48 min. The central portion of each peak of 280-nm absorbance was collected and lyophilized. Each lyophilized fraction was dissolved in 0.5 ml of deionized water. Protein concentrations of all RP-HPLC fractions were estimated spectrophotometrically. We used a value of $A_{280nm}^{0.10\%} = 1.1$ for an extinction coefficient. This was the average value of the extinction coefficient of the four ammonium sulfate fractions. A sample of about 10 μg of protein was used for gel electrophoresis, and 2 μg of protein was used for assays of inhibitory activity.

Assays of α-Amylase Inhibitors

Inhibitory activities were determined using four different α-amylases: human salivary α-amylase (Sigma Chemical Co., St. Louis, MO), porcine pancreatic α-amylase (Sigma Chemical Co.), α-amylase 1 of *Sitophilus oryzae* (rice weevil), and α-amylase of *Tribolium confusum* (confused flour beetle). The insect α-amylases were isolated following the method of Baker (1987). Amylase activities were determined according to the procedure of Bernfeld (1955). Amylase inhibitor assays were performed at the optimal pH of each amylase, using acetate buffer at pH 5.0 for insect α-amylases (Baker 1987) and phosphate buffer at pH 6.9 for human salivary and porcine pancreatic α-amylases. One unit of amylase and an inhibitor sample to be assayed were mixed in 0.2 ml of buffer and incubated at 37°C for 10 min. Then 0.5 ml of a 1% starch solution was added. After 5 min at 37°C, the reaction was stopped by adding 1 ml of dinitrosalicylic acid reagent. One amylase unit was defined as the amount of enzyme that liberated 1 mg of maltose during the 5-min reaction. One unit of inhibitor activity was defined as the amount of inhibitor that caused 50% inhibition of 1 unit of amylase.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in a high-molarity Tris buffer (Fling and Gregerson 1986). Nondenaturing gel electrophoresis was performed according to the procedure described by Davis (1964) using a pH 8.8 separating gel. Bromphenol blue was used as the tracking dye.

Protein Concentration

Protein concentrations of the crude extract, the heat-soluble proteins, and the four ammonium sulfate fractions were determined by the method of Lowry et al (1951). Bovine serum albumin was used as a standard.

RESULTS

Fractionation of Wheat Albumin Inhibitors by Ammonium Sulfate Precipitation

Figure 1 shows electrophoregrams of the wheat crude extract, the heat-soluble proteins, and the four ammonium sulfate fractions. Lanes A–F in panel I show that numerous high-molecular-weight polypeptides were removed by heating the crude extract and that the apparent molecular mass of the heat-soluble proteins was ~14 kDa. The heat-precipitated proteins appear to correspond to proteins with high mobilities (relative mobility [Rm] > 0.33) in nondenaturing gel electrophoresis (lanes A and B, panel II), whereas the heat-soluble proteins have relatively low mobilities. The four ammonium sulfate fractions have different polypeptide patterns, but several of the polypeptides are present in more than one ammonium sulfate fraction (lanes C–F in panel I). This is especially clear in the nondenaturing electrophoresis system (lanes C–F in panel II). For example, fraction AS 0.5–1.0 (lane D in panel II) contains four distinct bands with Rm values of 0.17, 0.24, 0.30, and 0.33. Fraction AS 1.0–1.5 (lane E) also exhibits four bands but is more enriched in 0.17 and 0.24 mobility proteins, whereas fraction AS 1.5–2.5 (lane F) basically contains only the 0.17 and 0.24 mobility proteins.

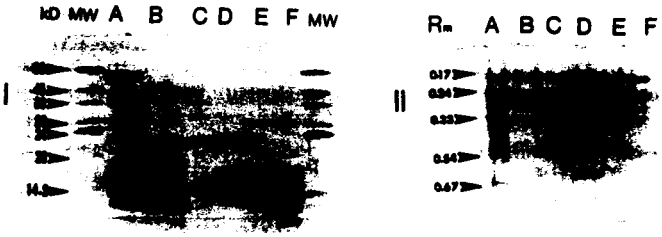

Fig. 1. Gel electrophoresis of wheat protein fractions. Panel I is by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and panel II is by nondenaturing polyacrylamide gel electrophoresis. The samples for lanes A and B were crude extract and heat-soluble proteins, respectively. Lanes C, D, E, and F represent ammonium sulfate fractions AS 0–0.5, AS 0.5–1.0, AS 1.0–1.5, and AS 1.5–2.5, respectively. Rm is mobility relative to bromphenol blue. MW = molecular weight.

Protein concentrations and inhibitory activities of the crude extract, heat-soluble proteins and the four ammonium sulfate fractions were determined and used to calculate specific inhibitory activities (Fig. 2). The four ammonium sulfate fractions had rather different inhibitory patterns against the test α-amylases. The crude extract, heat-soluble material and three of the four ammonium sulfate fractions exhibited substantially higher specific activities toward the insect and human α-amylases than toward porcine α-amylase. AS 0–0.5 had the highest specific activity toward porcine pancreatic α-amylase, whereas AS 0.5–1.0 and AS 1.0–1.5 had the highest specific activities toward human salivary and insect α-amylases. The AS 1.5–2.5 fraction was notable, however, because it had the highest specific activity toward insect α-amylases relative to its specific activity toward human salivary α-amylase, and it had a low activity toward porcine α-amylase.

RP-HPLC Separation of Wheat α-Amylase Inhibitors

Figure 3 shows RP-HPLC elution profiles of the four ammonium sulfate fractions. RP-HPLC resolved each ammonium sulfate fraction into numerous peaks, and each ammonium sulfate fraction produced a different profile based on absorbance at 280 nm. Numbered peaks were collected for further study. Results of electrophoresis and assays of inhibitory activity of the individual HPLC peaks are shown in Figure 4 and Table I. Based on position of HPLC elution, electrophoretic mobility, and inhibitory activity, several correlations between peaks present in different ammonium sulfate fractions could be made (Table II). For example, each pair of HPLC fractions 5 and 16, 7 and 18, 8 and 19, 24 and 32, 25 and 33, 26 and 34, and 27 and 35 probably represents the same proteins.

A wide spectrum of inhibitory activity was exhibited by some of the individual proteins resolved by HPLC. For example, peaks 8 and 35 inhibited only rice weevil α-amylase, whereas peaks 17 and 18 inhibited both insect α-amylases but not the mammalian enzymes. No HPLC peaks were substantially more active as mammalian α-amylase inhibitors than as insect enzyme inhibitors.

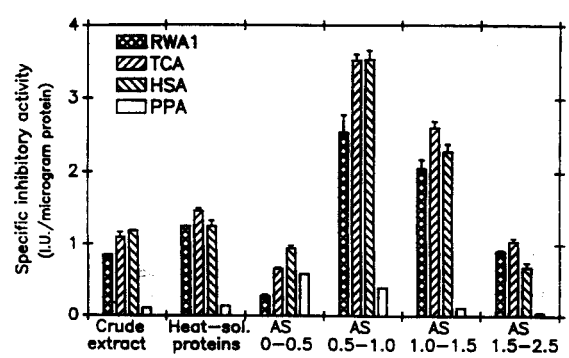

Fig. 2. Specific inhibitory activities of the wheat albumin fractions against rice weevil α-amylase 1 (RWA1), α-amylase of *Tribolium confusum* (TCA), human salivary α-amylase (HSA), and porcine pancreatic α-amylase (PPA). Specific activity is expressed as the number of inhibitor units per microgram of protein. Data are the mean values ± range ($n = 2$) except for PPA data, which are single determinations. AS = ammonium sulfate. I.U. = international unit.

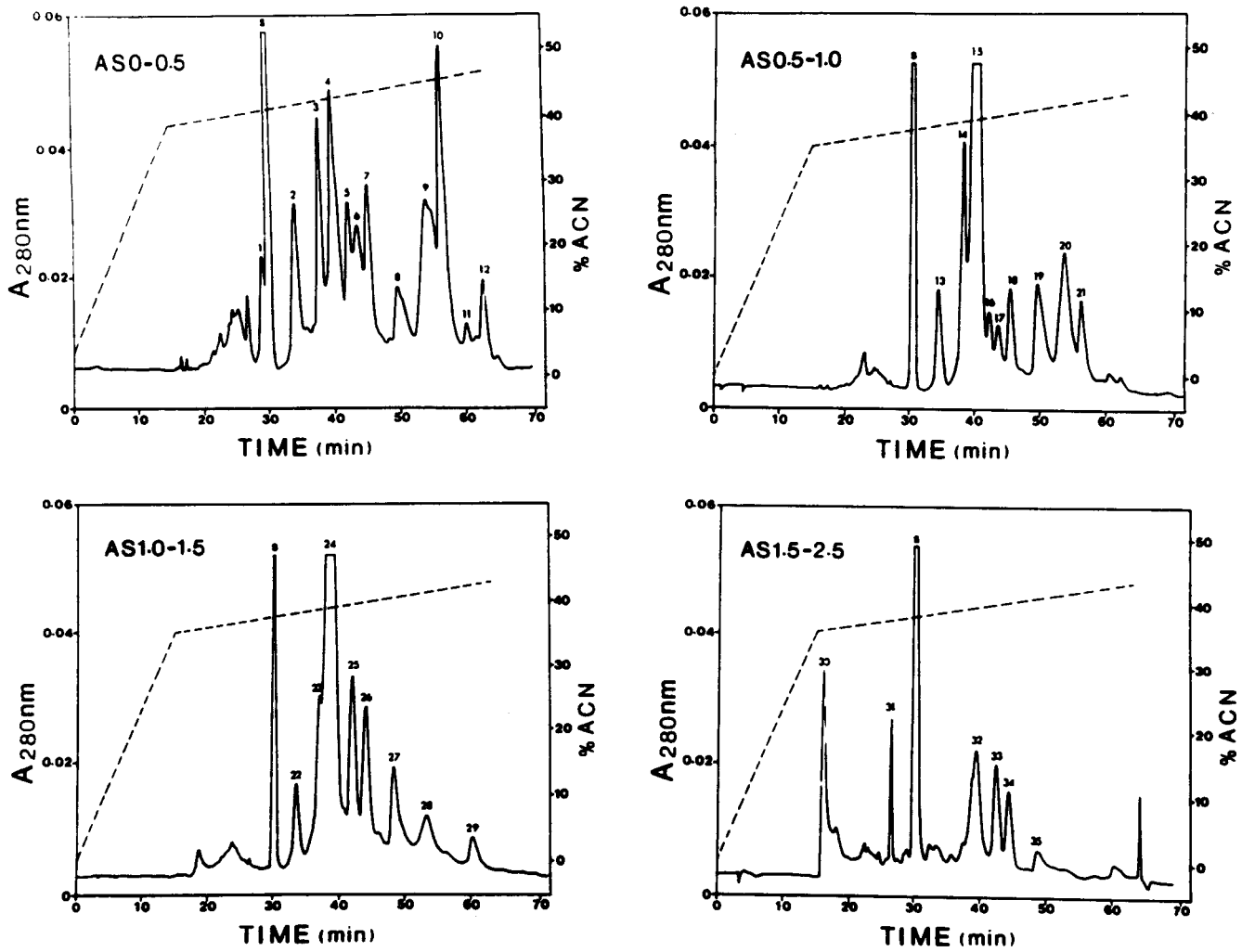

Fig. 3. Reversed-phase high-performance liquid chromatography elution profiles of four ammonium sulfate (AS) fractions. The dashed lines are the acetonitrile (ACN) gradients. Peak S is due to an unknown nonprotein component.

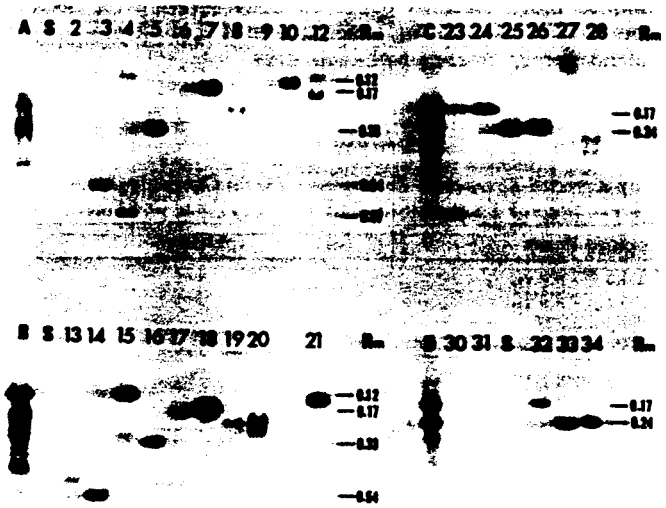

Fig. 4. Nondenaturing gel electrophoresis of reversed-phase high-performance liquid chromatography peak fractions. Lanes A, B, C, and D represent ammonium sulfate fractions AS 0–0.5, AS 0.5–1.0, AS 1.0–1.5, and AS 1.5–2.5, respectively. Rm is mobility relative to bromphenol blue. S represents an unknown nonprotein component.

Effect of Combining HPLC Peaks on Inhibitory Activity

As mentioned above, fraction AS 0–0.5 had the highest specific activity against porcine pancreatic α-amylase among the four ammonium sulfate fractions (Fig. 2). After RP-HPLC, no inhibitory activity toward the porcine enzyme was observed for any single peak (Table I). However, when peaks 1 to 10 were combined, the inhibitory activity against porcine pancreatic α-amylase was observed again (Table III). To investigate this phenomenon, we conducted a series of mixing experiments. We found that three HPLC fractions (either peaks 4, 5, and 10 or peaks 4, 7, and 10) could be combined to produce strong and selective inhibitory activity against porcine α-amylase. Peaks 4 and 10 are essential, but either peak 5 or peak 7 can be combined with them to enhance inhibitory activity. Note in Figure 4 and Table I that peak 4 contains two polypeptide chains and has strong inhibitory activity toward both insect and human salivary α-amylases, whereas each of peaks 5, 7, and 10 appears to be a single polypeptide and has low inhibitory activities against the α-amylases tested.

DISCUSSION

We have demonstrated that RP-HPLC, which separates proteins on the basis of hydrophobic interactions among protein, matrix, and mobile phase (Bietz 1986, Gooding 1986), is useful for separating wheat α-amylase inhibitors. RP-HPLC resolved the ammonium sulfate fractions into numerous peaks, and their biological activities were conserved (Table I). Some of the peaks exhibited single bands in nondenaturing gel electrophoresis, which indicates that they were highly purified. Thus, RP-HPLC appears to be a powerful purification technique for wheat α-amylase in-

hibitors.

Precipitation by ammonium sulfate is typically the first step in preparing wheat α-amylase inhibitors. However, precipitation usually is performed at a single ammonium sulfate concentration. We investigated the fractionation of the wheat albumin fractions by precipitating at several ranges of ammonium sulfate concentration and also studied the inhibitory properties of each ammonium sulfate fraction using two mammalian and two insect α-amylases as test enzymes.

The heat-soluble wheat protein fractions prepared by ammonium sulfate precipitation varied in their inhibitory pattern against vertebrate and invertebrate α-amylases. Three of the four fractions were substantially more effective against insect and human α-amylases than against porcine α-amylase. The first fraction was similar in specific inhibitory activity against all four α-amylases. Gatehouse et al (1986) identified one wheat protein fraction that inhibited porcine α-amylase and not red flour beetle amylase, but they also obtained three other fractions that exhibited the inverse relationship in relative inhibitory activity. Baker (1989) has purified from wheat two monomeric inhibitors of rice weevil α-amylase. Whether the fractions prepared in this study correspond to any of those prepared by Gatehouse et al (1986) or Baker (1989) is unknown. However, based on the relative mobilities on the nondenaturing gel (Fig. 4) and the inhibitory specificities of the HPLC fractions (Table III), we can correlate some of our HPLC fractions to wheat α-amylase inhibitor families identified previously (Silano 1987). For example, peaks 24 and 32 correspond to the 0.19 isoinhibitor family, which is a group of dimeric inhibitors of both insect and mammalian α-amylases, whereas peaks 25 and 33 and 26 and 34 belong to the 0.28 family, which includes monomeric inhibitors of insect α-amylases.

We found that several RP-HPLC peaks had unique α-amylase inhibitory activities individually and after combination with other peaks. In particular, HPLC peaks 7 and 18, 8 and 19, 25 and 33, 26 and 34, and 27 and 35 were the best insect α-amylase inhibitors and may be good candidates for antibiosis factors to use in genetic engineering programs for host-plant resistance. We also found that combining three peak fractions from AS 0–0.5 (either peaks 4, 5, and 10 or 4, 7, and 10) produced strong inhibition of porcine pancreatic α-amylase, despite the fact that not one of these peaks alone was inhibitory toward this enzyme. These results suggested that oligomeric inhibitors in the wheat albumin-globulin fraction are partially or totally separated into monomers by RP-HPLC and that synergistic effects on enzyme inhibition occur when certain monomeric proteins are combined. Mixing three of the wheat proteins (HPLC peaks 4, 5, and 10 or 4, 7, and 10) apparently leads to formation of an oligomeric inhibitor with high affinity for porcine pancreatic α-amylase. Proteins combined from either HPLC peaks 4, 5, and 10 or 4, 7, and 10 exhibited broad smeared bands on nondenaturing gel electrophoresis (data not shown), which indicated that the monomeric proteins were interacting with each other.

TABLE I
α-Amylase Inhibitory Activities of Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Fractions[a]

Ammonium Sulfate Fractions	RP-HPLC Fractions	Percent Inhibition[b]			
		RWA1	TCA	HSA	PPA
AS 0–0.5	3	19	0	5	0
	4	88	90	90	0
	5	23	0	5	0
	6	79	68	0	0
	7	49	8	0	0
	8	96	0	0	0
	9	8	0	0	0
	10	4	0	0	0
AS 0.5–1.0	14	84	91	86	0
	15	91	93	96	0
	16	nd	nd	nd	0
	17	90	89	0	0
	18	89	83	0	0
	19	93	27	10	0
	20	10	0	4	0
	21	0	0	4	0
AS 1.0–1.5	23	89	93	93	11
	24	94	95	96	5
	25	94	88	6	12
	26	90	89	0	8
	27	94	24	0	8
	28	10	0	0	0
AS 1.5–2.5	32	87	92	96	23
	33	84	82	0	22
	34	82	83	0	21
	35	90	0	0	8

[a] Inhibitory activities are expressed as percentage of inhibition of the amylase used. One amylase unit and approximately 2 μg of inhibitor protein were used in each assay.
[b] RWA1, rice weevil α-amylase 1 (*Sitophilus oryzae*); TCA, α-amylase of *Tribolium confusum* (confused flour beetle); HSA, human salivary α-amylase; PPA, porcine pancreatic α-amylase; nd, not determined.

TABLE II
Chromatographic Behavior, Electrophoretic Behavior, and α-Amylase Inhibitory Activity of High-Performance Liquid Chromatography (HPLC) Peaks from Different Ammonium Sulfate (AS) Fractions

HPLC Peaks	Time Eluted (min)	Rm[a]	Inhibitory Activity[b]			
			RWA1	TCA	HSA	PPA
5 (AS 0–0.5)	42	0.33	23	0	5	0
16 (AS 0.5–1.0)	42	0.33	nd	nd	nd	nd
7 (AS 0–0.5)	45	0.17	49	8	0	0
18 (AS 0.5–1.0)	45	0.17	89	83	0	0
8 (AS 0–0.5)	50	0.26	96	0	0	0
19 (AS 0.5–1.0)	50	0.26	93	27	10	0
24 (AS 1.0–1.5)	39	0.17	94	95	96	5
32 (AS 1.5–2.5)	39	0.17	87	92	96	23
25 (AS 1.0–1.5)	42	0.24	94	88	6	12
33 (AS 1.5–2.5)	42	0.24	84	82	0	22
26 (AS 1.0–1.5)	44	0.24	90	89	0	8
34 (AS 1.5–2.5)	44	0.24	82	83	0	21
27 (AS 1.0–1.5)	49	0	94	24	0	8
35 (AS 1.5–2.5)	49	nd	90	0	0	8

[a] Relative mobility on nondenaturing gel.
[b] RWA1, rice weevil α-amylase 1 (*Sitophilus oryzae*); TCA, α-amylase of *Tribolium confusum* (confused flour beetle); HSA, human salivary α-amylase; PPA, porcine pancreatic α-amylase; nd, not determined.

TABLE III
Inhibition of Porcine Pancreatic α-Amylase by Reversed-Phase High-Performance Liquid Chromatography Peak Fractions of Ammonium Sulfate 0–0.5 and Some Combinations[a]

Combined Peaks	Inhibition
1,2,3,4,5,6,7,8,9,10	+
2,3,4,5,6,7,8,9,10	+
2,3,4,5,6,7,8,9	—
4,5, 7,8,9,10	+
5, 7,8,9,10	—
4, 8,9,10	—
4,5, 10	+
4, 7, 10	+
4,5	—
4, 7	—
4, 10	—
5, 7	—
5, 10	—
7, 10	—
4	—
5	—
7	—
10	—

[a] One amylase unit and 1 μg of each peak fraction protein were used in each assay. + means more than 80% inhibition, and — means no inhibition.

A similar observation regarding reconstitution of inhibitory activity from purified polypeptides has been reported by Gomez et al (1989). Their data showed that a wheat oligomeric inhibitor of α-amylase from the yellow mealworm, *Tenebrio molitor*, could be reconstituted when either of two sets of "CM proteins" (CM2, CM3A, and CM16 or CM1, CM3D, and CM17) was prepared. No single CM protein (i.e., monomeric polypeptide) showed inhibitory activity against the same α-amylase from *T. molitor*. Apparently, the species selectivity of the α-amylase inhibitors from wheat can be determined by the oligomeric composition.

Multimeric inhibitors, such as tetramers, presumably were formed both in our reconstitution experiments and the experiments of Gomez et al (1989). This type of α-amylase inhibitor is less well studied than are monomeric and dimeric wheat α-amylase inhibitors (Deponte et al 1976, O'Connor and McGeeney 1981, Buonocore et al 1985, Baker and Lum 1989). RP-HPLC is a valuable method for isolating subunits of oligomeric wheat inhibitors, including tetrameric inhibitors. The technique may allow detailed studies about changes in α-amylase inhibitory activity following oligomer formation.

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